CBP ms.19467 Revised – part A

Impact of photoperiod manipulation on day/night changes in melatonin, sex steroids and vitellogenin plasma levels and spawning rhythms in Senegal sole, *Solea senegalensis*

Catarina Oliveira[a], Evaristo Mañanós[b], Jesus Ramos[b], Francisco Javier Sánchez-Vázquez[c*]

[a] *CIMAR/CCMAR, Algarve University, Campus de Gambelas, 8005-139 Faro, Portugal.*
ccoliveira@ualg.pt
[b] *Department of Fish Physiology and Biotechnology, IATS/CSIC, 12595 Castellón, Spain.*
evaristo@iats.csic.es and ramos@iats.csic.es
[c] *Department of Physiology, Faculty of Biology, Murcia University, 30100 Murcia, Spain.*

Running title: *Photoperiod effect on reproduction rhythms in sole*

* Corresponding Author:
Dr. F. Javier Sánchez Vázquez
Department of Physiology, Faculty of Biology
University of Murcia, Campus Espinardo
30 100 Murcia, SPAIN
javisan@um.es

Abstract

Photoperiod and temperature are known as the main synchronizers of seasonal reproduction in fish. This paper studied the role of photoperiod on the synchronization of F1 Senegal sole's reproduction rhythms. Fish were maintained under constant short-photoperiod (9L:15D) from the winter solstice onwards (experimental group) or under naturally-changing photoperiod (control group), and water temperature naturally oscillated in both groups. Blood samples were collected during reproduction season at pre-spawning (March), spawning (April) and

post-spawning (May) to determine the endocrine status. Spawning events and egg quality parameters were also monitored. The results revealed a significant increase in nocturnal melatonin concentration from March to May in the control group, while in the experimental group such seasonal change did not occur. As to plasma levels of vitellogenin, testosterone, estradiol and 11keto-testosterone, differences between groups were found mostly in March, while in April and May levels were often similar. Spawning was observed in both groups, although the experimental group started slightly earlier and also finished earlier than the control group, perhaps as a result of the increase in sex steroids and VTG observed at pre-spawning. Briefly, reproduction rhythms persisted in the absence of the natural lengthening of photoperiod, although photoperiod manipulation altered the seasonal modulation of melatonin, increased sex steroids and vitellogenin at pre-spawning, and slightly advanced the timing of spawning.

Keywords: melatonin, photoperiod, sex steroids, *Solea senegalensis*, spawning, vitellogenin

1. Introduction

Reproduction in fish is rhythmic and timed to guarantee the maximum survival of the offspring. Environmental cues, such as photoperiod and temperature cycles, synchronize the internal timing system that controls breeding (Bromage et al., 2001). The pineal organ is a possible candidate to be the mediator between environmental cycles and the brain-pituitary-gonad (BPG) axis, controlling reproduction timing. This organ transduces daily and seasonal cycles into a neurohormonal signal, producing melatonin (MEL) and working both as a clock and a calendar in order to provide the BPG axis with environmental information, although the exact pathway of its action is yet unknown (Falcón et al., 2007).

Sex steroids are produced in the gonads and act both on the brain and anterior lobe of the pituitary to control the production of luteinizing hormone (LH) (Yaron et al., 2003), while vitellogenin (VTG) is a protein that specifically appears in the blood of sexually maturing female of oviparous and ovoviviparous animals during vitellogenesis in response to circulating estrogens (Specker et al., 1994). Thus, plasma concentrations of both sex steroids and VTG are good indicators of the status of the BPG axis and hence the reproductive condition of the animals.

The Senegal sole, *Solea senegalensis*, is a species that has incited increasing interest in both aquaculture and basic research during the past decades. However, several questions of sole aquaculture still need to be optimized, including the control of reproduction (Agulleiro et al., 2006; Dinis et al., 1999). The spawning season of this species is observed mainly in spring, although a secondary season may occur in autumn (Anguis et al., 2005). Sex steroids and VTG exhibited annual rhythms with high concentrations during the pre-spawning period in spring, and a secondary, less pronounced increase around autumn (García-López et al., 2006a; Guzmán et al., 2008). The seasonal rhythms of melatonin have also been assessed recently, with temperature modulating its nocturnal production (Vera et al., 2007). Temperature also seemed to play a key role in reproduction timing, since Senegal sole deprived of its naturally oscillating seasonal thermo cycle did not sustain reproduction rhythms (García-López et al., 2006b; Oliveira et al., 2009), probably due to the absence of clock/calendar information provided by melatonin rhythms. However, to date there are no studies focusing on the effect of the seasonal photoperiod cycle and such phenomenon requires attention in order to better understand the synchronization of the seasonal reproduction rhythms in this species and thus optimize breeding protocols.

The aim of this research was to investigate the role of photoperiod in the synchronization of reproduction rhythms in Senegal sole subjected to two different photoperiodic conditions: naturally fluctuating vs. constant short-photoperiod from winter solstice onwards. The levels of MEL, sex steroids (testosterone, T, 17β-estradiol, E_2, and 11keto-testosterone, 11KT) and VTG were measured during pre-spawning, spawning and post-spawning periods , as well as spawning and egg quality parameters.

2. Material and Methods

2.1. Animals and housing

Twenty-five Senegal sole (*Solea senegalensis* Kaup, 1858) from the research centre "Instituto de Acuicultura de Torre la Sal" (IATS) (40°6'N, 0°9'E, Castellón, Spain) were used for this research. They were from an F1 generation and had a mean body mass of 1577.7 ± 77.5 g and a mean body length of 47.5 ± 0.6 cm. They were divided in an experimental group, reared under a constant short photoperiod (L9:D15) from winter solstice onwards (n=13, 7 females and 6 males) and a control group, reared under natural photoperiod conditions (n=12, 6 females and 6 males). Both groups were reared indoors in three cylindrical fiberglass tanks

(4000 L, 1 m depth, 4.9 m^{-2}) equipped with egg collectors, which were checked daily to verify the presence of eggs. The water was provided with a constant flow of running marine water from the Mediterranean Sea, with a natural annual cycle of temperature (ranging between 13.5 and 18 ºC during the sampling months) and a salinity of 37‰. Fish were fed *ad libitum* in the morning five days a week, using a dry pellet feed (ProAqua) and natural food, consisting of chopped fresh mussels and frozen squid. All sole were individually labeled with an internal passive integrated transponder (PIT) tag system, enabling identification of each fish in repeated samplings.

Handling of the fish was in accordance with the European Union Directive (EEC, 1986) for the protection of animals used for experimental and other scientific purposes.

2.2. Experimental design

To determine the influence of the photoperiod seasonal cycle on the regulation of Senegal sole reproduction, fish from the experimental group were submitted to a constant short photoperiod from the 21st December until the reproduction season. This photoperiod was obtained by covering the two experimental group tanks with a lightproof cover, and illuminating the water surface with a florescent bulb of 11 w and 240 v (19E1000, RS Components, Ltd, Northants, UK) with a maximum light intensity of 500 lux. The tank containing the control group was just covered with a raffia cloth, which provided a natural photoperiod, with maximum light intensity of 600 lx at the water surface during the day. Sampling was performed at three critical moments of the reproductive cycle according to Guzmán et al., (2008): at pre-spawning (7/8 March), at full spawning (23 April) and at post-spawning (27/28 May). At each time interval each fish was blood sampled at Mid-Dark (MD) and at Mid-Light (ML), to detect daily fluctuations on hormone levels. During the dark phase, sampling was carried out under a dim red light and after covering the fish head with aluminum foil. Soles previously anesthetized with 2-phenoxyethanol (0.3 mL L^{-1}), were individually weighed and identified with a “pit-tag” lector, and then sampled by caudal puncture, with 1 mL heparinized syringe. Blood was collected in heparinized eppendorf tubes on ice to later be centrifuged (3 000 g, 4 ºC, 15 min), and then plasma was separated into three aliquots (for MEL, sex steroids and VTG measurement) and frozen at -80 ºC.

Spawning events were monitored daily during reproduction season for both groups, and on days spawning took place eggs were collected and weighed. As a measure of egg quality, buoyant eggs were separated from non-buoyant eggs in graduated cylinders filled with sea water (salinity 37‰) and buoyancy % calculated. Buoyant eggs from each spawn

were incubated for 48 h to determine hatching %. Total number of eggs produced daily was related to the female biomass of the group at that time, to calculate the relative daily fecundity (# of eggs kg^{-1}). Total fecundity for each group was obtained at the end of the spawning season and related to the female biomass of the group, to calculate the relative total fecundity (total # of eggs kg^{-1}) (Guzmán et al., 2008).

2.3. Data analysis

Melatonin analysis

MEL levels in plasma samples were measured by a Radioimmunoassay commercial Kit (Melatonin Direct RIA, Biosource, Belgium), with a lower limit of quantification (LLOQ) of 2 pg/mL, as described by Oliveira et al. (2007).

Vitellogenin and Sex Steroid ELISA

To assess plasma VTG, T and E_2 in females, and T and 11KT in males, samples were analysed in the facilities of the Institute of Aquaculture of Torre la Sal (IATS) (Castellón, Spain), by enzyme-linked immunosorbent assay (ELISA) using protocols previously validated for Senegal sole plasma samples (Guzmán et al., 2008).

Statistical analysis

Statistical tests were performed using SPSS®. Data are expressed as mean ± standard error of the mean (S.E.M.). Plasma concentrations of MEL, VTG and sex steroids, were subjected to one-way ANOVA, followed by a Duncan's post-hoc test (with a degree of significance of $p<0.05$) to determine the existence of significant differences between sampling points. Finally, a t-student test was performed ($p<0.05$) to detect the existence of differences in spawning quality parameters between the two groups.

3. Results

In the short photoperiod group, plasma MEL concentrations measured at MD were statistically higher than the values registered at ML and remained similar during the three months sampling took place (oscillating slightly from 230.5 ± 66.1 to 287.6 ± 48.5 pg/mL). However in the control group plasma MEL values measured in the MD-soles in May (274.0 ± 53.8 pg/mL) were significantly higher than in March (138.6 ± 34.1 pg/mL) (ANOVA,

165 Duncan's test, $p<0.05$), showing a seasonal increase in nocturnal concentrations (fig. 1).
166 However, there were no significant differences between groups at any sampling point.
167 Concerning the spawning rhythms, the experimental group spawned eight times from
168 7^{th} to 29^{th} April, while the control group spawned also eight times but later, from 18^{th} April to
169 27^{th} May (fig. 2, table 1). In what refers to spawn weight per day, egg buoyancy (%) and daily
170 mean fecundity there were no significant differences registered between groups ($p<0.05$ t-
171 student test) although the values slightly differed (table 1). In all cases no fertilized eggs were
172 obtained.
173 Regarding VTG and sex steroids in general, the profiles differed between the two
174 groups mainly at pre-spawning, being very similar at full and post-spawning. In females,
175 plasma VTG concentrations in the experimental group were significantly higher at MD in
176 March (pre-spawning) (6.2 ± 0.6 mg/mL) than at ML in April (full spawning) (3.1 ± 0.6
177 mg/mL), while for the control group, MD values in March (1.5 ± 0.4 mg/mL) were lower than
178 all samplings in April and May (mean values 5.3 ± 0.6 and 4.5 ± 0.5 mg/mL, respectively)
179 (fig 3). Between groups, significantly different values were only observed at MD in March
180 (ANOVA, Duncan's test, $p<0.05$). For the sex steroids in females the pattern was very
181 similar, with most differences between the two groups being found in March. In the case of T
182 (fig. 4a), the experimental group values were significantly higher at ML in March (1.5 ± 0.2
183 ng/mL) than in all the other sampling points, and there were also higher concentrations
184 observed at March MD when compared to April MD (0.7 ± 0.2 and 0.3 ± 0.1 ng/mL,
185 respectively). Between groups, values just differed at ML in March. With regard to E_2, March
186 ML plasma concentration in the experimental group were higher (7.4 ± 1.2 ng/mL) than in the
187 following months, while March MD values (5.7 ± 0.6 ng/mL) just differed from April MD
188 and May ML. Moreover, in April and in May MD and ML values differed, thus showing
189 day/night differences on these months. In the control group, day/night differences were also
190 observed in April, with higher values being detected at ML (4.6 ± 0.8 ng/mL against 1.5 ± 0.5
191 at MD ng/mL) (ANOVA, Duncan's test, $p<0.05$). Between groups, different plasma
192 concentrations of E_2 were observed in the sampling points ML in March and both at MD and
193 ML in May.
194 In the case of males, sex steroid concentrations (T and 11KT) only showed different
195 levels between groups in March. With regard to T rhythm, in both the experimental and
196 control groups the plasma concentrations registered in March were significantly higher than in
197 the following months (3.0 ± 0.3 and 2.4 ± 0.2 ng/mL, respectively) (fig. 5a), just with the
198 difference that in the experimental group day/night differences were detected in March. With

regard to 11KT rhythms, only the experimental group showed higher levels in March, (18.3 ± 1.5 ng/mL), while control group values remained similar during the three sampling months (ANOVA, Duncan's test, $p<0.05$) (fig. 5b).

4. Discussion

The group of Senegal sole subjected to constant short photoperiod from the winter solstice onwards failed to show the seasonal increase in nocturnal melatonin seen in the control group. However, these fish successfully spawned during the same period (spring) as the control group but few days earlier, perhaps as a result of the increase in sex steroids and VTG observed at pre-spawning. These animals were able to time the spawning season in the absence of photoperiod information during three months prior to reproduction, which may suggest they used other environmental cycles (seasonal changes in water temperature) or the presence of a free-running reproduction rhythm.

The seasonality of reproduction in fish is known to be mediated by the naturally changing pattern of photoperiod and temperature during the year, and, depending on the species, either one or the other factor (or both combined) is the main cue to synchronize these rhythms (Bromage et al., 2001; Falcón et al., 2010). As a long-day breeder, in sole the naturally lengthening day towards spring should play a key role in triggering the onset of vitellogenesis, steroidogenesis and spawning. According to recent investigations (Vera et al., 2007) sole kept during the whole year in conditions of controlled temperature suffered a disruption of the MEL rhythms, lost their annual environmental information and failed to spawn. Furthermore, under such constant temperature conditions, the animals lost their annual sex steroid rhythms, preventing spawning (García-López et al., 2006b; Oliveira et al., 2009). Based on all the above evidence, it seems that the main cue Senegal sole uses is temperature, since the animals successfully synchronized their reproduction season without seasonal information on the photoperiod. In spite of the stated in Bromage et al., (2001) considering photoperiod as the principal determinant of maturation in flatfish, salmonids and in most other teleosts, in Senegal sole we could be facing a different strategy, since this fish has evolved interesting ways to synchronize spawning. In the case of the Eurasian perch, a species which also spawns during spring, seasonal photoperiodic variations were required to induce and control the reproductive cycle, although temperature also played an important role (Migaud et al., 2006). For species such as sea bass (Bayarri et al., 2004; Prat et al., 1999; Zanuy et al.,

1995) or cod (Davie et al., 2003; Hansen et al., 2001), studies have demonstrated a profound effect of photoperiod on gonadal maturation, fecundity, sex steroid levels and spawning. In contrast, both cues combined are required by cyprinids for the accurate synchronization of reproduction (Hontela et al., 1990). However, to the best of our knowledge, there is no evidence of other teleosts from temperate latitudes using temperature as the main determinant of the timing of reproduction.

The MEL concentrations reached during the night in the control group were similar to those seen in the same species under natural thermo and photoperiod conditions for the same months (Vera et al., 2007). However, in the absence of the natural lengthening of the photoperiod, the seasonal rise in nocturnal melatonin could not be detected within the time frame of the samplings performed, thus suggesting that both photo and thermo information are being transduced by the pineal organ, modulating the melatonin rhythm. In the case of the sea bass, in different seasons of the year with similar values of temperature, the rise in melatonin levels differed greatly according to the photoperiod (García-Allegue et al., 2001), suggesting a role for both these environmental factors in the modulation of nocturnal melatonin.

The rhythm of VTG in females was similar in the two groups, except during pre-spawning, and its concentrations remained high in all three months, generally similar to those described before by Guzmán et al., (2008). According to this author, Senegal sole showed high circulating VTG levels during several months, suggesting vitellogenesis in this species as a long term process. In our study, females from both groups also presented high concentrations of VTG during the three months, suggesting on-going vitellogenesis during this period. With regard to sex steroids in females, both E_2 and T rhythms were similar to values observed for the VTG: differences between groups were observed at pre-spawning, but during spawning and post-spawning values were similar in the case of T and also differed in May for E_2. In males the pattern was also similar, with higher concentrations of sex steroid registered at pre-spawning in the experimental group. The values recorded in our study were similar to those described in previous studies on sex steroids in Senegal sole (García-López et al., 2006a; Guzmán et al., 2008; Oliveira et al., 2009), although such comparisons should be treated carefully because the rhythms of steroids vary greatly between years and are related with the natural variations in water temperature. The fact that the experimental group presented significantly higher concentrations of VTG and sex steroid concentrations during pre-spawning period could be responsible for the earlier spawning period observed in this group.

The appearance of day/night differences only in some samplings of each steroid studied (T: in March for both females and males of the experimental group; E_2: in April and May for the experimental group and April for the Control group) may be due to a phase shift of the daily rhythms, a change in its timing. However, the existence of day/night differences in sex steroids strongly suggests that these concentrations do not remain static during the day. Curiously enough, in all points day/night differences were observed, concentrations were always higher during the day, with the exception of E_2 in May, when the experimental group seemed to invert this tendency, presenting higher values during the night, suggesting an alteration of the daily rhythm, and consequently of the daily peak. However, more time points would be necessary to confirm such result.

The absence of egg fertilization observed in the present study is a common phenomenon in the F1 generation of Senegal sole, as described previously in the studies of Guzmán et al., (2008) and Agulleiro et al., (2006) and is probably due to a lack of synchrony between females and males at the time of spawning. Due to this fact, in the present paper it was not possible to determine whether the photoperiodic manipulation could be affecting the final spawn quality. However, the other parameters accessed such as fecundity or egg buoyancy rate seemed similar between two groups, not showing any tendency to an alteration of quality.

5. Conclusions

In conclusion, Senegal sole deprived from photoperiod information during the last three months prior to reproduction, sustained nocturnal MEL concentration from March to May, abolishing the seasonal rise observed in natural conditions. However, animals maintained their VTG and sex steroid rhythms, although at a higher level at pre-spawning compared with the control group. As a result, the spawning rhythm persisted, though it was slightly advanced under constant short-photoperiod, stressing the possibility of manipulating spawning season in fish farms. These findings bring about new insights on the understanding of reproduction rhythms of Senegal sole, which represents a bottle neck for aquaculture production of this species.

6. Acknowledgments

This work was financed by the MEC (project *Reprosolea* AGL2004-07984-CO2-01/ACU granted to FJSV and project AGL2006-13777-C03-01 to EM) and by SENECA (Project 05690/PI/07 to FJSV). CO received a FPI Fellowship from the MEC. The authors would also

like to thank Caridad Meseguer and José Maria Gúzman for their help during sample collection and laboratory sample analysis.

References

Agulleiro, M.J., Anguis, V., Cañavate, J.P., Martínez-Rodríguez, G., Mylonas, C.C., Cerdá, J., 2006. Induction of spawning of captive reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 257, 511-524.

Anguis, V., Cañavate, J.P., 2005. Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. Aquaculture 243, 133-145.

Bayarri, M.J., Rodríguez, L., Zanuy, S., Madrid, J.A., Sánchez-Vázquez, F.J., Kagawa, H., Okuzawa, K., Carrillo, M., 2004. Effect of photoperiod manipulation on the daily rhythms of melatonin and reproductive hormones in caged European sea bass (Dicentrarchus labrax). Gen. Comp. Endocrinol. 136, 72-81.

Bromage, N., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63-98.

Davie, A., Porter, M.J.R., Bromage, N.R., 2003. Photoperiod manipulation of maturation and growth of Atlantic cod (*Gadus morhua*). Fish Physiol. Biochem. 28, 399-401.

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture 176, 27-38.

EEC, 1986. Council Directive 86/609 EEC for the protection of animals used for experimental and other scientific purposes. Official Journal L358, 1-28.

Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends Endocrinol. Metab. 18, 81-88.

Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469-482.

García-Allegue, R., Madrid, J.A., Sánchez-Vázquez, F.J., 2001. Melatonin rhythms in european sea bass plasma and eye: influence of seasonal photoperiod and water temperature. J. Pineal Res. 31, 68-75.

García-López, A., Anguis, V., Couto, E., Canario, A.V.M., Cañavate, J.P., Sarasquete, C., Martínez-Rodríguez, G., 2006a. Non-invasive assessment of reproductive status and cycle of sex steorid levels in a captive wild broosdstock of senegalese sole *Solea senagalensis* (Kaup). Aquaculture 254, 583-593.

García-López, A., Pascual, E., Sarasquete, C., Martínez-Rodríguez, G., 2006b. Disruption of gonadal maturation in cultured Senegalese sole *Solea senegalensis* Kaup by continuous light and/or constant temperature regimes. Aquaculture 261, 789-798.

Guzmán, J.M., Norberg, B., Ramos, J., Mylonas, C.C., Mañanós, E., 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). Gen. Comp. Endocrinol. 156, 285-297.

Hansen, T., Karlsen, Ï., Taranger, G.L., Hemre, G.I., Holm, J.C., Kjesbu, O.S., 2001. Growth, gonadal development and spawning time of Atlantic cod (*Gadus morhua*) reared under different photoperiods. Aquaculture 203, 51-67.

342 Hontela, A., Stacey, N.E., 1990. Cyprinidae. In: Munro, A.D., Scott, A.P., Lam,T.J. (Eds.),
343 Reproductive seasonality in teleosts: environmental influences, CRC Press, Boca Ratón, FL, pp.
344 53-77.
345 Migaud, H., Wang, N., Gardeur, J.-N., Fontaine, P., 2006. Influence of photoperiod on reproductive
346 performances in eurasian perch *Perca fluviatilis*. Aquaculture 252, 385-393.
347 Oliveira, C., Vera, L.M., López-Olmeda, J.F., Guzmán, J.M., Mañanós, E., Ramos, J., Sanchez-
348 Vazquez, F.J., 2009. Monthly day/night changes and seasonal daily rhythms of sexual steroids
349 in Senegal sole (*Solea senegalensis*) under natural fluctuating or controlled environmental
350 conditions. Comp. Biochem. Physiol. A 152, 168-175.
351 Prat, F., Zanuy, S., Bromage, N., Carrillo, M., 1999. Effects of constant short and long photoperiod
352 regimes on the spawning performance and sex steroid levels of female and male sea bass. J.
353 Fish Biol. 54, 125-137.
354 Specker, J.L., Sullivan, C.V., 1994. Vitellogenesis in fishes: status and perspectives. In: Davey, K.G.,
355 Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Endocrinology, National Research Council,
356 Ottawa, pp. 304-315.
357 Vera, L.M., Oliveira, C., López-Olmeda, J.F., Ramos, J., Mañanós, E., Madrid, J.A., Sánchez-
358 Vázquez, F.J., 2007. Seasonal and daily plasma melatonin rhythms and reproduction in senegal
359 sole kept under natural photoperiod and natural or controlled water temperature. J. Pineal Res.
360 43, 50-55.
361 Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish
362 gonadotropins. Int. Rev. Cytol. 225, 131-185.
363 Zanuy, S., Prat, F., Carrillo, M., Bromage, N.R., 1995. Effects of constant photoperiod on spawning
364 and plasma 17ß-oestradiol levels of sea bass (*Dicentrarchus labrax*). Aquat. Living Resour. 8,
365 147-152.
366
367 **Figure Captions**
368
369 Figure 1. Plasma concentrations of melatonin during spawning season (values expressed as
370 mean ± S.E.M.) in Senegal sole exposed to an experimental short photoperiod (mid-dark:
371 black spotted bars; mid-light: white spotted bars) and exposed to a natural oscillating
372 photoperiod (mid-dark: black bars; mid-light: white bars). Different letters (a, b, c), indicate
373 groups with significant differences between sampling points (ANOVA, Duncan's test
374 $p<0.05$).
375
376 Figure 2. Spawning events observed during the spawning season in a group of Senegal sole
377 exposed to an experimental short photoperiod (black bars) and another group exposed to a
378 natural oscillating photoperiod (grey bars) (values expressed as g of eggs day $^{-1}$).
379
380 Figure 3. Plasma concentrations of vitellogenin during spawning season (values expressed as
381 mean ± S.E.M.) in Senegal sole females exposed to an experimental short photoperiod (mid-
382 dark: black spotted bars; mid-light: white spotted bars) and exposed to a natural oscillating

photoperiod (mid-dark: black bars; mid-light: white bars). Different letters (a, b, c) indicate groups with significant differences between sampling points (ANOVA, Duncan's test $p<0.05$).

Figure 4. Plasma concentrations of sex steroids (testosterone, T and estradiol, E_2) during spawning season (values expressed as mean ± S.E.M.) in Senegal sole females exposed to an experimental short photoperiod (mid-dark: black spotted bars; mid-light: white spotted bars) and exposed to a natural oscillating photoperiod (mid-dark: black bars; mid-light: white bars). Different letters (a, b, c, d, e) indicate groups with significant differences between sampling points (ANOVA, Duncan's test $p<0.05$).

Figure 5. Plasma concentrations of sex steroids (testosterone, T and 11keto-testosterone, 11-KT) during spawning season (values expressed as mean ± S.E.M.) in Senegal sole males exposed to an experimental short photoperiod (mid-dark: black spotted bars; mid-light: white spotted bars) and exposed to a natural oscillating photoperiod (mid-dark: black bars; mid-light: white bars). Different letters (a, b, c, d) indicate groups with significant differences between sampling points (ANOVA, Duncan's test $p<0.05$).

401 Table 1. Spawning characteristics for both control and experimental groups

	Control group	Experimental Group
# females	6	7
Sex ratio (f:m)	1:1	1:0.86
# spawns	8	8
1st spawning day	18-04-2007	07-04-2007
last spawning day	27-05-2007	29-04-2007
Daily fecundity	12.50±2.86	9.96±1.89
Total fecundity	100.32	79.67
Egg buoyancy (%)	16.30±5.04	9.75±6.86
Hatching rate (%)	0	0

402
403

Figure 1

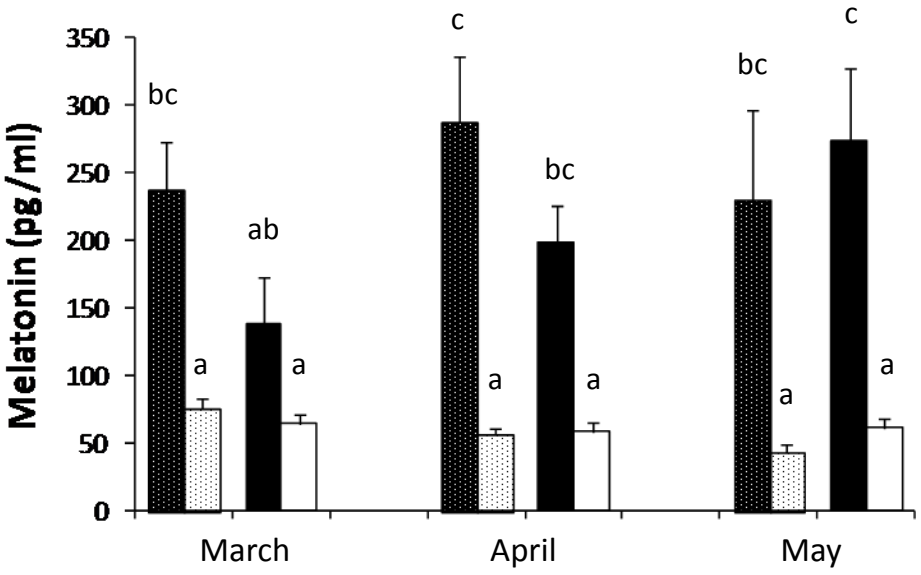

Melatonin (pg/ml)
350
300
250
200
150
100
50
0
bc
a
ab
a
c
a
bc
a
bc
a
c
a
March
April
May

Figure 2

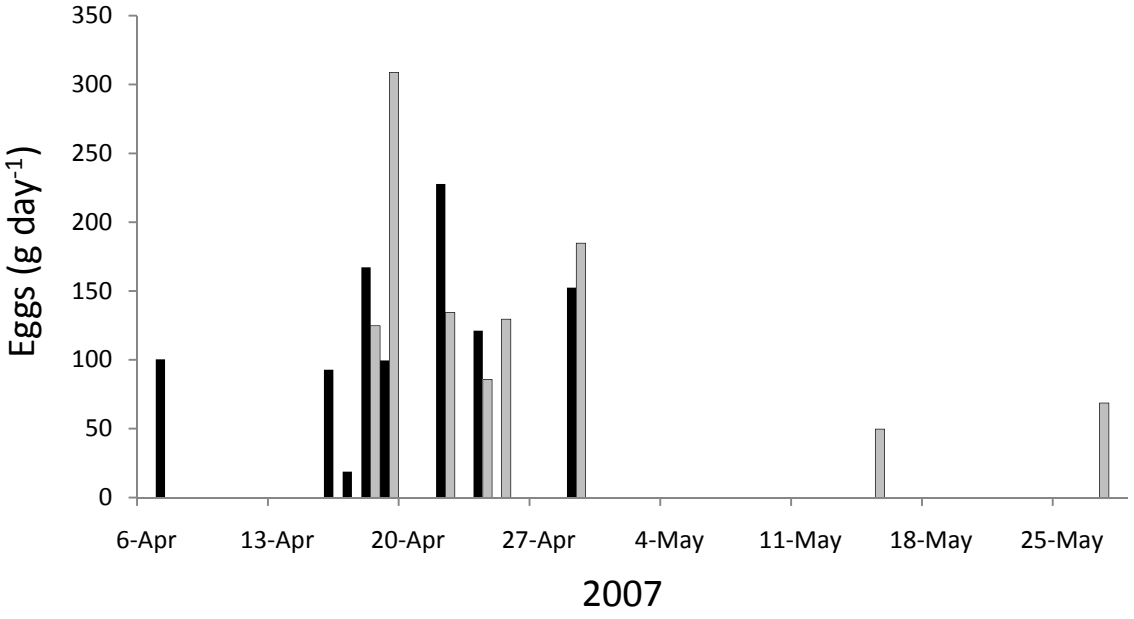

350
300
250
200
150
100
50
0
Eggs (g day-1)
6-Apr
13-Apr
20-Apr
27-Apr
4-May
11-May
18-May
25-May
2007

Figure 3

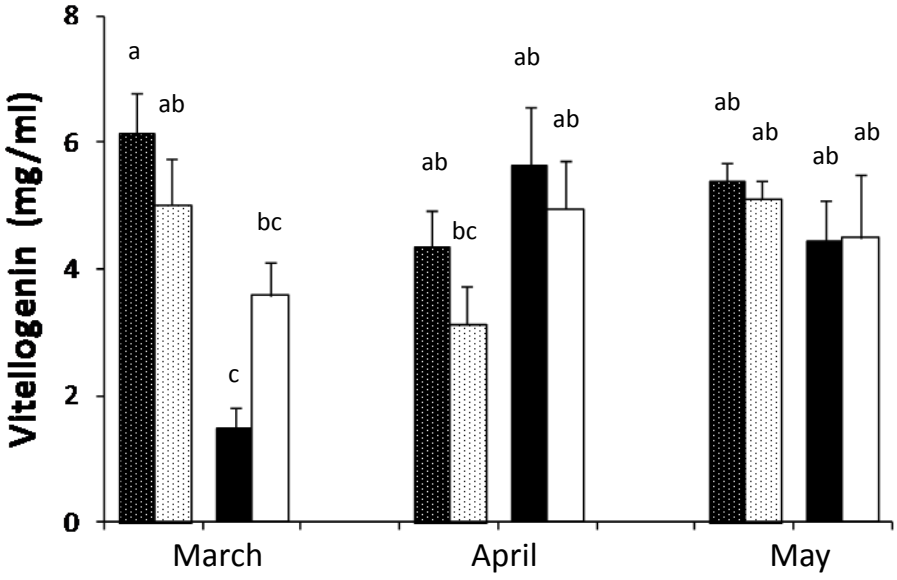

Vitellogenin (mg/ml)
8
6
4
2
0
a
ab
c
bc
ab
bc
ab
ab
ab
ab
ab
ab
March
April
May

Figure 4

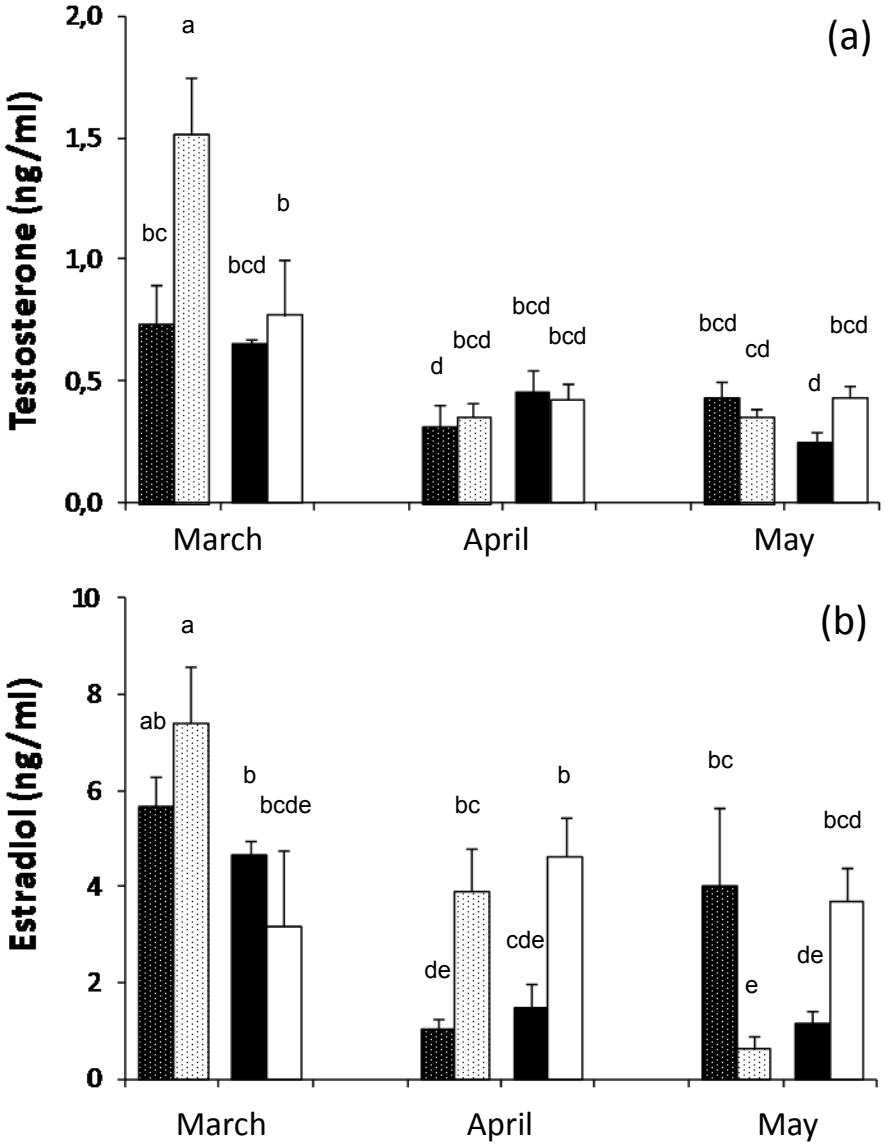
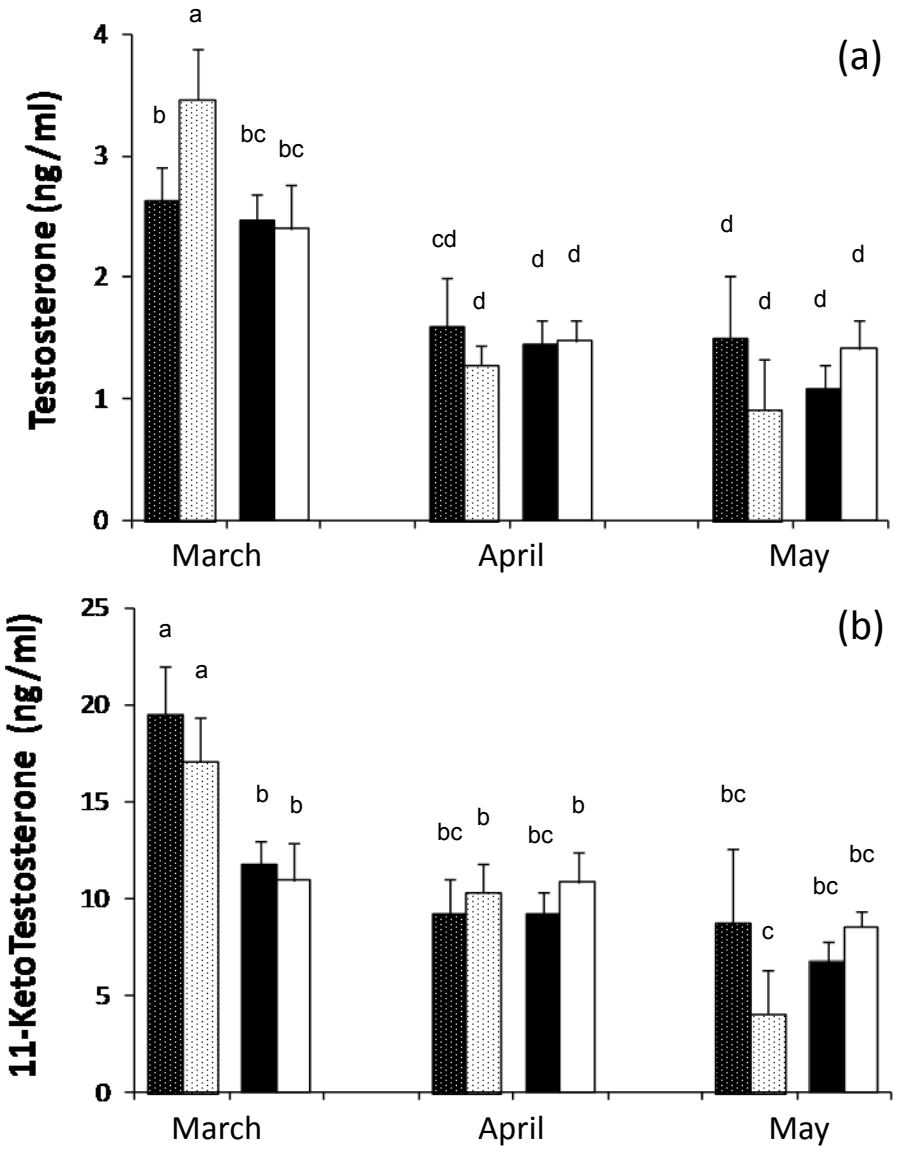

(a)
Testosterone (ng/ml)
2,0
1,5
1,0
0,5
0,0
a
bc
b
bcd
d
bcd
bcd
bcd
bcd
cd
bcd
d
March
April
May
(b)
Estradiol (ng/ml)
10
8
6
4
2
0
a
ab
b
bcde
bc
de
b
cde
bc
e
bcd
de
March
April
May

Figure 5


(a)
Testosterone (ng/ml)
4
3
2
1
0
a
b
bc
bc
cd
d
d
d
d
d
d
d
March
April
May
(b)
11-KetoTestosterone (ng/ml)
25
20
15
10
5
0
a
a
b
b
bc
b
bc
b
bc
c
bc
bc
March
April
May